Laboratory Characterization of Noroviruses Identified in Specimens from Military Health System Beneficiaries During an Outbreak in Germany, 2016–2017

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Although infectious gastroenteritis is one of the most common illnesses associated with military deployments, such illness is also relatively frequent in service members who are not deployed and in civilian populations in developed countries.[1] Military personnel have a particularly high risk of acute gastroenteritis due in part to their close proximity or contact with one another in closed environments which facilitate transmission of viral pathogens.[2] Although gastroenteritis can be bacterial, viral, or parasitic in nature, norovirus has been identified as one of the top five etiologic agents of gastroenteritis among military populations.[3] Norovirus is classified as a non-enveloped, single-strand RNA virus that is highly contagious. The incubation period for norovirus illness is 24–72 hours.[4] Symptoms of norovirus infections typically last 1–3 days and rarely require hospitalization.[4] Six genogroups of norovirus have been identified; of these, genogroup I (GI), genogroup II (GII), and genogroup IV (GIV) affect humans.[5] A total of 25 different genotypes have been identified within the three human genogroups, although since 2002, variants of the GII.4 genotype have been most commonly identified among norovirus outbreaks.[6]

In 2016, among residents of the Federal Republic of Germany, norovirus incidence reported during the winter season was unusually high and reported earlier than usual.[7] In November 2016, at least 14,872 laboratory-confirmed cases were reported in Germany, representing almost twice the median number of cases reported (7,810 cases) in the same month over the past 5 years.[7] During the 2016–2017 norovirus season (October–March, Epidemiological Weeks 39–13), a total of 79,378 cases were reported by the Robert Koch Institute.[8] The total case count was greater than the counts during each of the previous four seasons (42,621 cases in 2015–2016; 67,646 cases in 2014–2015; 59,587 cases in 2013–2014; and 66,783 cases in 2012–2013). The largest number of norovirus cases in recent years in Germany was the 95,575 cases reported during the 2011–2012 season.[8]

The Robert Koch Institute reported an emerging norovirus recombinant strain (GII.P16–G.II.2) during the 2016–2017 outbreak. The strain was identified in nine federal states across Germany by sequencing two open reading frames: ORF1 (polymerase) and ORF2 (capsid).[7] Herd immunity in the German civilian population was likely attained against previously circulating norovirus strains; however, Niendorf et al. concluded that the acquired immunity against previous strains may not have been effective against the emerging variant strain, further propagating the outbreak.[7] This study characterizes norovirus isolates from Military Health System (MHS) beneficiaries which corresponded temporally and geographically with the 2016–2017 outbreak in Germany.

MATERIALS AND METHODS

During the 2016–2017 norovirus season in Germany, stool samples from MHS beneficiaries with gastroenteritis were collected at various military treatment facilities (MTFs) in the U.S. European Command (EUCOM) between August 2016 and March 2017; one sample collection site was unspecified. Samples were sent to Landstuhl Regional Medical Center (LRMC) for testing using the FilmArray® Gastrointestinal Panel (BioFire®). Potential exposure location data for affected individuals were retrospectively identified using the Armed Forces Health Longitudinal Technology Application (AHLTA). Of all samples received by LRMC during this surveillance period, a total of 41 tested positive for norovirus. Norovirus sequencing was not available at LRMC at the time of this study. Therefore, all 41 samples were shipped in April 2017 to the Naval Health Research Center (NHRC), San Diego, CA, for sequencing.

Upon receipt at NHRC, stool samples were initially diluted to 20% in phosphate-buffered saline without Ca^{2+}/Mg^{2+}. The resulting solution was processed for total RNA content using the QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). RNA samples were subjected to a one-step, real-time, TaqMan®, RT-PCR assay developed by the Centers for Disease Control and Prevention's CaliciNet Program for the simultaneous detection of norovirus GI and GII. Norovirus-positive samples were genotyped based on sequences obtained from the amplification of partial regions of ORF1 (polymerase) and/or ORF2 (capsid). Phylogenetic analysis was performed using the Lasergene Molecular Biology Suite in conjunction with National Center for Biotechnology Information Basic Local Alignment Search Tool.

RESULTS

All 41 samples received by NHRC had tested positive for norovirus GI or GII at LRMC, using a commercial multiplex gastrointestinal PCR panel cleared by the Food and Drug Administration. Of the 41 samples, a total of 33 tested positive for norovirus at NHRC. Two samples tested positive for norovirus GI, and 31 tested positive for norovirus GII. Eight samples were found to be negative for both norovirus GI and GII by two different molecular assays at NHRC. To determine whether the sensitivity of the different testing platforms was the underlying factor for the discrepant testing results between sites, one positive and

TABLE 1. Norovirus sequences and potential exposure location in Military Health System beneficiaries during the 2016–2017 winter season (N=31)

Norovirus genotype	Country (N, %)
GI.P2–GI.2	Belgium (1, 3%)
GI.3A	U.S. (1, 3%)
GII.P16–GII.2	Germany (7, 22%); Belgium (2, 6%); Italy (1, 3%); Hungary (1, 3%)
GII.P16–GII.4 Sydney	Germany (5, 16%)
GII.2	Germany (1, 3%)
GII.P21–GII.3	Belgium (1, 3%)
GII.P4 NewOrleans 2009–GII.4 Sydney 2012	Germany (3, 9%)
GII.4 Sydney	Germany (2, 6%); Montenegro (1, 3%)
GII.Pe–GII.4 Sydney	Germany (1, 3%)
GII.P7–GII.6	Germany (3, 9%); Belgium (1, 3%)
Total no. of samples	Germany (22), Belgium (5), Italy (1), Montenegro (1), U.S. (1), Hungary (1)

FIGURE. Phylogenetic analysis of norovirus GI and GII sequences based on ORF1 (polymerase) and ORF2 (capsid) regions
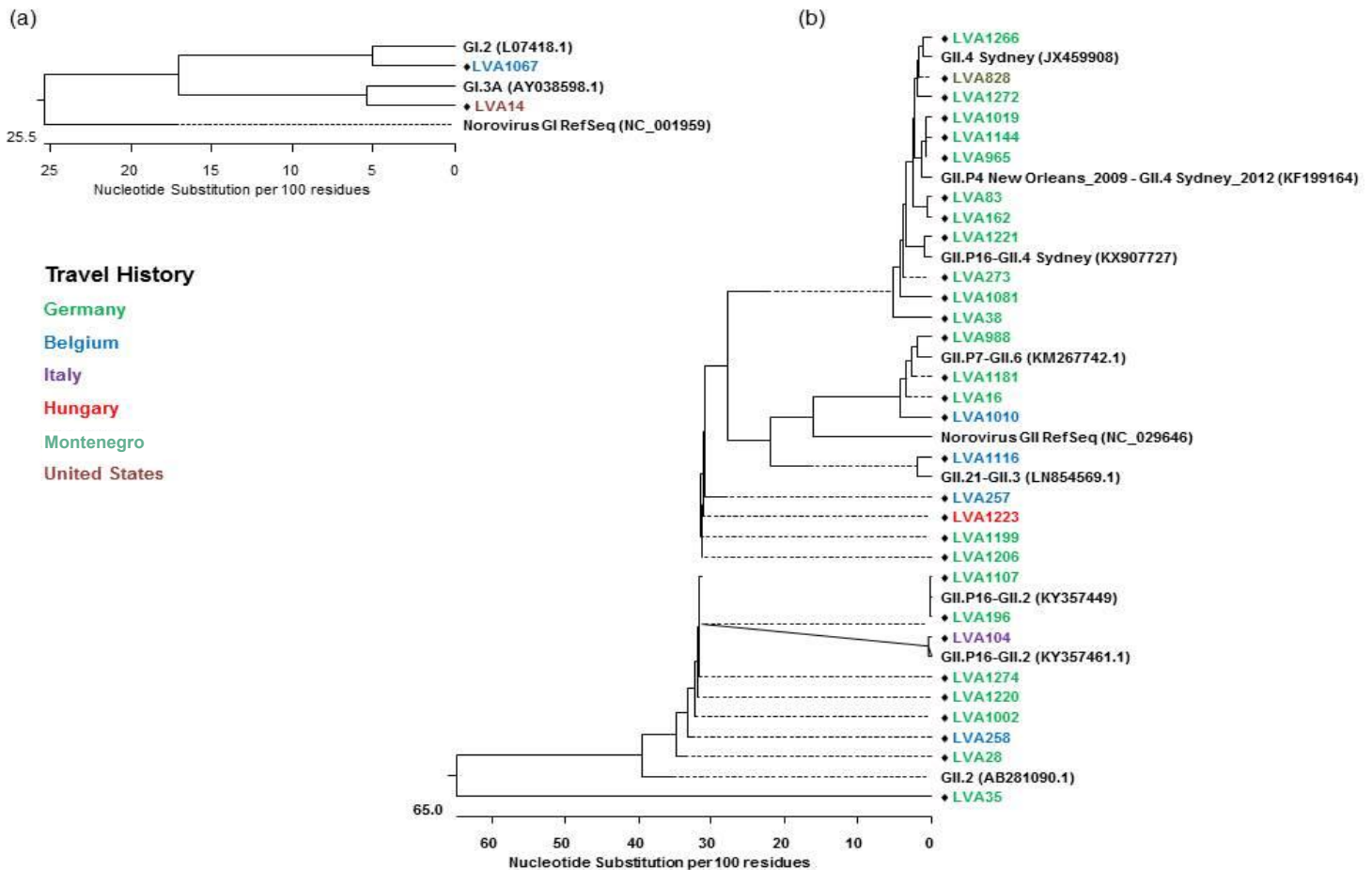
Note: Reference strains of norovirus genotypes are listed with their respective accession numbers. Landstuhl Regional Medical Center samples are color-coded according to the subject's travel history. (a) Norovirus GI phylogenetic analysis. (b) Norovirus GII phylogenetic analysis.

TABLE 2. NHRC sequencing results for Military Health System beneficiary norovirus specimen samples obtained from LRMC during 2016–2017 (N=41)

Group	No. of samples (N)	% distribution of NHRC results	% distribution by genotype per Niendorf et al.
GI.P2–GI.2	1	3%	0%
GI.3A	1	3%	4%
GII.2	1	3%	1%
GII.P16–GII.2	11	35%	45%
GII.P16–GII.4 Sydney	5	16%	10%
GII.4 Sydney	3	10%	10%
GII.Pe–GII.4 Sydney	1	3%	
GII.P4 NewOrleans2009–GII.4 Sydney2012	3	10%	9%
GII.P7–GII.6	4	13%	4%
GII.P21–GII.3	1	3%	2%
Total no. of samples sequenced	31	100%	-
Failed[a]	2	-	N/A
Negative[b] by real-time and conventional PCR	8	-	-
Total no. of samples received	41	-	-

NHRC, Naval Health Research Center; LRMC, Landstuhl Regional Medical Center

[a]NHRC was able to identify norovirus GI or GII in the sample but was not able to sequence it successfully for genotype assignment.

[b]The sample was negative by real-time RT-PCR and RT-PCR for norovirus GI and/or GII.

four negative specimens were processed at NHRC through the same multiplex PCR system as at LRMC. The results confirmed the one positive and four negative findings previously obtained by NHRC using the TaqMan®, RT-PCR assay **(Table 1)**. Phylogenetic analysis was completed on the 31 GII-positive samples **(Figure)**. Norovirus recombinant genotypes were characterized in 25 out of the 31 specimens analyzed **(Table 2)**. The most abundant recombinant strains were GII.P16–GII.2 (35%) and GII.P16–GII.4 Sydney (16%). These data are comparable to the findings from a study by Niendorf et al., which assessed the norovirus strains circulating in Germany during the winter season of 2016–2017. That study reported nearly half of the civilian samples genotyped were GII.P16–GII.2.[7] A variety of other norovirus genotypes were also found by Niendorf et al. to be co-circulating during the same time frame but were detected less frequently than these dominant strains.[7]

Among the 31 individuals who had norovirus GII samples sequenced, 22 had histories of travel to, or lived in, Germany. Because of the limited data available, it was difficult to distinguish between travel history and previous residence. The remaining individuals reported travel or residence in other countries: Belgium, Italy, Montenegro, U.S., and Hungary **(Table 1)**.

EDITORIAL COMMENT

Eight of the 41 specimens that had tested positive at LRMC tested negative for both norovirus GI and GII at NHRC. The manufacturer of the multiplex PCR kits recommends that specimens be processed and tested as soon as possible (within 4 days of collection) to ensure accurate test results. Discrepant multiplex PCR panel results between the two testing sites could be due to a variety of factors, including RNA degradation during storage at and/or transit from LRMC to NHRC. Furthermore, the possibility of initial false-positive results at LRMC could not be ruled out.

The LRMC laboratory received samples collected from patients located throughout Europe, providing a range of travel histories and likely contributing to the variety of norovirus strains obtained in the sequencing results. However, the finding that 7 (32%) of 22 specimens from Germany were positive for the emerging recombinant strain (GII.P16–GII.2) suggests that the outbreak in the German population this past winter was genetically similar to the incident norovirus cases in the MHS beneficiary population in Germany. More than 50% of the samples tested yielded at least one of the two ORF genes characterized in the outbreak strain identified in the study by Niendorf et al. These observations exemplify the extent of integration between the MHS beneficiary population and the local German population and the impact on outbreak dynamics.

Transmission within the MHS beneficiary community and the local German community likely occurred through common exposure points. Locations such as workplaces, restaurants, grocery stores, and day care facilities could have been frequented by both military personnel and the local German population. The results highlight the importance of understanding disease risk and transmission characteristics within the local population in Germany because it is likely that for certain diseases, risk will similarly affect the MHS beneficiary population living in the German community. Moreover, timely collection of samples and rapid diagnosis of an etiologic agent in conjunction with other biosurveillance activities may help to provide early warning for future gastrointestinal outbreaks. Beginning in late 2017, LRMC will have enhanced gastrointestinal surveillance capability that can inform development of appropriate Force

Health Protection guidance and aid in prevention of disease transmission as well as mitigate possible operational impacts.

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